BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
G01N 33/574, C07K 15/28
C12Q 1/37

A1

(11) International Publication Number: WO 93/20447

(43) International Publication Date: 14 October 1993 (14.10.93)

(21) International Application Number: PCT/US93/02967

(22) International Filing Date: 31 March 1993 (31.03.93)

(30) Priority data:
07/860,901 31 March 1992 (31.03.92) US

(71) Applicant: THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK [US/US]; P.O. Box 9, Albany, NY 12201-0009 (US).

(72) Inventor: ZUCKER, Stanley ; 68 South Pond Lane, Smithtown, NY 11787 (US).

(74) Agent: SACK, Alan, M.; Hoffmann & Baron, 350 Jericho Turnpike, Jericho, NY 11753-1317 (US).

(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: DIAGNOSTIC TESTS MEASURING GELATINASE/INHIBITOR COMPLEXES FOR DETECTION OF AGGRESSIVE AND METASTATIC CANCER

(57) Abstract

Diagnostic agents and methods for detecting the presence of metastatic activity in biological samples such as plasma are disclosed. The agent and method preferably immunologically detect matrix metalloproteinases in complexed form with endogenous inhibitors of MMP's. A kit for detecting the metalloproteinases is also disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SK	Slovak Republic
CI	Côte d'Ivoire	KZ	Kazakhstan	SN	Senegal
CM	Cameroon	LI	Liechtenstein	SU	Soviet Union
CS	Czechoslovakia	LK	Sri Lanka	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	UA	Ukraine
DK	Denmark	MG	Madagascar	US	United States of America
ES	Spain	ML	Mali	VN	Viet Nam
FI	Finland	MN	Mongolia		

DIAGNOSTIC TESTS MEASURING GELATINASE/INHIBITOR COMPLEXES FOR DETECTION OF AGGRESSIVE AND METASTATIC CANCER

BACKGROUND OF THE INVENTION

The present invention relates to improvements in the diagnosis of metastic disease. In particular, the invention relates to techniques for detecting the presence of proteolytic enzymes associated with metastatic disease.

Although a cure for most forms of cancer remains elusive, early detection and treatment have historically provided the best prognosis. Considerable effort over the years has been directed to developing diagnostic tests which give an indication of the presence of metastatic disease at an early stage.

Recently, a relationship between matrix metalloproteinases (MMP's) and metastasis has been suggested. MMP's are a family of closely related metal-dependent endopeptidases secreted by mesenchymal cells. For example, gelatinase A (MMP-2 or 72 kDa gelatinase/type IV collagenase) and gelatinase B (MMP-9 or 92 kDa gelatinase/type IV collagenase) have been identified as playing a major role in cancer invasion and metastasis. Stromelysin and PUMP (Putative Metalloproteinase), metalloproteinases with broader substrate specificity, are also thought to participate in the metastatic process. Three of these metalloproteinases have been identified in mammalian plasma.

Metastasis is a complex sequence of events in which malignant cells detach and disseminate from a primary tumor. The malignant cells invade adjacent tissue, penetrate into blood and lymphatic vessels, circulate to distant sites and eventually attach to and penetrate tissues in distant organs, thereby proliferating the malignancy. An important part of the metastatic process is the degradation of extracellular basement membranes by various proteolytic enzymes.

Basement membranes are collagen-containing connective tissues which form a tough continuous sheet and separate the various cell layers such as the epithelial, endothelial and parenchymal cells from interstitial connective tissue.

Proteolytic enzymes associated with the metastatic process are found circulating in the form of activated enzyme (free enzyme), latent free enzyme and complexed with endogenous proteinase inhibitors. Only the active forms, however, digest the connective tissue substrates. The body's own natural defense mechanisms rapidly inactivate MMP's by complexing the enzymes with specific tissue inhibitors of metalloproteinases (TIMP's). Such inhibitors found in the tissues and circulating in the plasma include TIMP-1, TIMP-2 and also alpha-2 macroglobulin which is primarily in blood.

In spite of the relationship between elevated levels of certain destructive proteases and the presence of metastatic disease, it has been difficult to use the relationship to provide an accurate indication of metastatic disease. In the past, it has only been possible to detect free or activated forms of the metalloproteinases, leaving a substantial portion of the inactivated enzymes complexed with inhibitors undetected. Recently it has been shown, however, that latent gelatinase A forms complexes with TIMP-2 and latent gelatinase B forms complexes with TIMP-1. Thus, these gelatinases can exist outside the cell in complexed forms with TIMPs.

U.S. Patent No. 4,677,058 discloses purifying and detecting type IV collagenase antigens from malignant tumor cells. Similarly, U.S. Patent No. 4,808,528 discloses antibodies specific to type IV collagenase enzyme antigens. U.S. Patent No. 4,816,400, a division of the '058 patent, supra, discloses immunological determination of typ IV

collagenase antigens using polyclonal and monoclonal antibodies. None of these references, however, disclose detecting collagenase enzyme-inhibitor complexes associated with metastatic disease.

In spite of the investigation of the role of metalloproteinases in metastatic disease, the total amount of MMP's being released and thus the actual metastatic activity has not been detectable. Indeed, due to the usually rapid inactivation of activated MMP's by TIMP's, measuring only free, circulating MMP's would fail to indicate the presence of many underlying diseases. Determining whether or not the complexes formed between MMP's and TIMP's form a more reliable and a sensitive diagnostic tests has yet to be investigated.

It is therefore an object of the present invention to provide highly specific and reliable diagnostic agents and methods to determine the presence of metastatic disease based on measuring matrix metalloproteinase inhibitor complex levels.

Other and further objects and advantages of the present invention will become apparent to those skilled in the art from a consideration of the following description taken together with the accompanying figure.

SUMMARY OF THE INVENTION

The present invention includes diagnostic agents and methods useful in the detection of metastatic activity in biological samples such as human plasma. The invention includes a first substance capable of immunologically reacting with enzymes broadly described as matrix metalloproteinases (MMP's). Such MMP's include gelatinase A 72 kDa type IV collagenase /gelatinase (MMP-2), gelatinase B 92 kDa

type IV collagenas /gelatinase (MMP-9), str melysin (MMP-3), putative metallopr teinase (PUMP), partial breakdown products of these proteins and combinations thereof. The invention further includes a second substance which is immunologically reactive with endogenous inhibitors of the tissue-degrading enzyme metalloproteinases or TIMP's.

The first immunologically-reactive substance is preferably a monoclonal antibody having specificity for one or more MMP enzymes or circulating breakdown products of MMPs. Examples of such antibodies include monoclonal murine anti-MMP-2, monoclonal murine anti-MMP-9 and monoclonal murine anti-MMP-3, such that set forth by Bergmann, et al. J. Clin. Chem. Clin. Biochem 27, 351-359 (1989) or Cell Tech Lmt. (Slough, England). Alternatively, antibodies such as rabbit polyclonal antibodies to native MMP's or peptide components such as peptide sequences of the native MMP's may be used.

The second immunologically responsive substance is preferably an antibody having specificity for endogenous inhibitors of MMP's, TIMP's or other associated proteins which bind to MMP's in plasma or tissue. For example, polyclonal rabbit anti-TIMP-1 or polyclonal anti-TIMP-2 as described by Carmichael, et al. Proc. Natl. Acad Sci. USA, 83:2407-2411 (1986) or the N.I.H. (Bethesda, MD) are examples of such substances. Alternatively, monoclonal antibodies to TIMP's may be selected.

The present invention also includes a method of detecting metastatic activity in a biological sample and a diagnostic kit. The method includes contacting a diagnostic agent, such as that set forth above, with the biologic sample and measuring the total amount of matrix metalloproteinases present and TIMP complexes uncovered to d termine wheth r metastatic dis as is pres nt.

The diagnostic agent, method and kit of the present invention can be included as part of various immunoassay techniques, particularly ELISA and most preferably sandwich-type ELISA assays. Alternative immunoassay techniques such as immunoblotting, immunofluorescent, radio-immunoassay, fluorescence detection and/or enzyme assay methods are also contemplated.

As a result of the present invention, significantly more accurate determinations of metastatic activity are obtained by detecting not only free metalloproteinases and breakdown products of MMP's, but also complexes of the enzymes formed with inhibitors. This is a dramatic improvement over methods which only detect free or activated MMP's and completely missed those enzymes complexed to the inhibitor molecules. In addition, the assays described herein provide independent verification of disease. Thus, the results provided can supplement other tests and provide additional data not obtained with other tests.

For a better understanding of the present invention, reference is made to the following description, taken together with the accompanying figure, and its scope will be pointed out in the appended claims.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 graphically demonstrates the results of immunological assays carried out using sandwich-type ELISA techniques to detect metastatic activity in patients using the present invention.

DETAILED DESCRIPTION OF THE INVENTI N

The diagnostic agent and method of the present invention are based on the premise that high levels of certain metalloproteinases complexed to TIMP's in clinical samples strongly correlate with an underlying metastatic disease. Thus, while not necessarily indicating a particular type of cancer, the diagnostic agent and method of the present invention provide a means of indicating or affirming the presence of underlying metastatic disease in the biologic fluids of suspected patients.

Blood and/or plasma are the most common biologic fluids assayed for diagnostic tests. For purposes of the present invention, the term biologic fluids shall also include but not be limited to plasma, serum, tissue samples, aspirates, urine and tissue fluids. It is contemplated that the inventive diagnostic agent and method provide an indication of metastatic activity from any sample containing metalloproteinases in both free and complexed form, even in amounts as low as the nanogram per milliliter level.

The identification of metalloproteinases in clinical samples is preferably carried out using immunologic techniques. The immunologic techniques center around the use of specific antibody-antigen reactions which indicate a response to only specific antigens, in particular, those enzymes associated with metastatic disease. Within this genre of tissue-degrading enzymes are gelatinase A 72 kDa type IV collagenase/gelatinase (MMP-2), gelatinase B 92 kDa IV collagenase/gelatinase (MMP-9), stromelysin (MMP-3), putative metalloproteinase (PUMP's), and breakdown products of the proteinases and combinations thereof. For diagnostic purposes, it is of importance to note that the recognition of these antigenic substances is in both the free and complex d form.

In order to achieve this result, the invention includes a first substance having immun specificity to free metalloproteinases and complexed metalloproteinases. In a preferred embodiment, monoclonal antibodies are prepared to have the desired immunospecificity. For example, monoclonal antibodies may be obtained from hybridomas obtained from mice immunized by injection of 72 kDa progelatinase/type IV procollagenase purified from human fibroblasts. See Birkedal-Hansen, et al. Biochemistry 27, 6751-6758 (1988). It is essential that the antibody bind to the specific metalloproteinases when it is complexed to TIMP. Many antibodies currently used can recognize free metalloproteinase, but they fail to bind to MMP in complexes with inhibitors as described in more detail below. A non-limiting list of suitable antibodies include murine monoclonal anti-human 72 kDa or 92 kDa type IV collagenase/gelatinase antibodies, or murine monoclonal antibodies to stromelysins. One method of producing these antibodies which recognize MMP's in complexes is to use MMP-TIMP complexes as the immunogen in mice or rabbits.

The inventive diagnostic agent also includes a second substance having particular specificity for the naturally-occurring tissue inhibitors of metallo-proteinases (TIMP's), including TIMP-1, TIMP-2, and alpha macroglobulin. These antibodies need to be reactive to TIMP's in complexes with MMP's.

Free TIMP-1 has a molecular weight of 28 kDa, but in complexes with MMP's, it is identified as approximately 95 kDa. Free TIMP-2 has a molecular weight of 22 kDa, but complexed with MMP's, it is identified as multiple components with molecular weights between 23 kDa to 150 kDa. Because gelatinase A and gelatinase B breakdown over time following activation or enzyme digestion, it is anticipated that lower molecular weight products of these

MMPs may circulate in complexes with TIMP's. Th molecular weights of TIMP's in plasma have been identified using immunoblotting techniques of non-reduced SDS-PAGE polyacrylamide gels.

Preferably, antibodies which achieve the necessary binding to complexed TIMP are prepared specific to MMP-TIMP complexes found in the biologic sample. For example, polyclonal antibodies to human progelatinase/pro-type IV collagenase can be produced in rabbits by laboratory procedures known in the art. A good source for the immunogens is to isolate MMP-TIMP complexes from human plasma using gelatin Sephrarose chromatography to bind these antigens and dimethyl sulfoxide to elute the complexed proteins from the solid phase. These antigens are mixed with Freund's Adjuvant to enhance antibody response and are injected subcutaneously in rabbits on 3-5 occasions over a period of 4-6 weeks. Further examples of such polyclonal antibodies include rabbit anti-TIMP-1, rabbit anti-TIMP-2 and anti-alpha-2-macroglobulin. Suitable antibodies are also available from commercial laboratories such as Cell Tech Lmt. (Slough, England).

In an alternative embodiment, monoclonal antibodies are prepared with specificity to the TIMP complexes such as MAC-015 from Cell Tech Lmt. Combinations of antibodies are also contemplated. The monoclonal antibodies can also be prepared using laboratory techniques known to those of ordinary skill in the art, such as that provided by Cooksley, et al. MATRIX 10:285-291, 1990, or from commercial laboratory sources such as Cell Tech Lmt. The antibodies employed in this assay could also react to a new immunogen consisting of peptide components derived from a portion of the TIMP molecule and a portion of the MMP molecule. Likewise, neoantigens could be produced as a result of the complexing of TIMP and MMP's, thus resulting in a unique

antigen that would be diagnostic of the formation of complexes.

A preferred immunologic means of detecting metalloproteinases is the Enzyme-Linked Immunosorbent Assay (ELISA) method, and in particular the sandwich-type ELISA format. This assay method includes introducing a biologic sample between a capture layer of antibodies and a detection layer of antibodies.

In this regard, diagnostic well plates such as Immulon II 96 well microtiter plates available from Dynatech, Alexandria, VA are first coated with a capture antibodies directed to MMP's such as rabbit polyclonal antibodies to human 72 kDa gelatinase or 92 kDa progelatinase/type IV procollagenase. The capture antibody is introduced into the wells in amounts ranging from about 10 μl to about 200 μl, with amounts of about 100 μl being preferred. The capture antibody is preferably diluted in a suitable buffer such as 0.1 M $NaHCO_3$, pH 9.0 to about 1:200 concentration prior to introduction into the wells.

Binding of the capture antibody to the well is carried out over a period of from about 4 to about 24 hours and preferably about 18 hours after inoculation at temperatures ranging from about 0 to about 10°C and preferably about 4°C. Unbound antibody is thereafter removed by vigorously inverting the plates. The wells containing the bound antibody are next bathed with a bovine serum albumin/ bicarbonate buffer to block excess binding sites on the wells. The bottom layer of the "sandwich" or capture layer is completed by washing the plate thoroughly with a buffer solution containing sodium phosphate, sodium chloride and Tween 20.

The ELISA technique further includes introducing a biologic sample such as human plasma nto the capture antibody layer. The samples are preferably prepared by being diluted in an incubation-suitable buffer to about a 1:10 concentration. One such solution contains 50 mM sodium phosphate, 0.1 M sodium chloride, 0.02% Tween 20 and 0.1% bovine serum albumin (BSA). The samples are placed in the well, incubated at a temperature ranging from about 25°C to about 37°C, and preferably at about 37°C for a time period of from about 1 hour to about 4 hours and preferably about one hour.

The final part of the ELISA "sandwich" is a detection layer containing an antibody specific to free TIMP-1 or TIMP-2 recognizing TIMP in complexes. The wells containing the capture antibody and sample are washed thoroughly before introducing a detection antibody into the well such as monoclonal murine anti-TIMP-1, anti-TIMP-2 antibodies or the like. The antibodies are preferably added after being diluted in an incubation buffer to about a concentration of about 1:4000.

The wells are incubated for about 1 hour and thereafter washed thoroughly and prepared using standard ELISA techniques known to the art, such as including the amplifying goat antibodies to mouse IgG and then alkaline-phosphatase conjugated to streptavidin. The wells are then washed and 100 microliters of substrate p-nitrophenyl phosphate in buffer is added to generate a color reaction which is read at A_{405}. The results of the assay are obtained using any suitable reading devise such as that available from BioTek of Winooski, VT. In addition, the optimal concentration of capture antibodies such as polyclonal rabbit anti-gelatinase A, and detecting antibodies such as monoclonal murine anti-TIMP-1, is determined using ch ck rboard titrati n. In this pr cedur ,

the concentrations of different antibodies and antigens are varied by serial dilution of the reagents to determine the concentration of each reagent, giving the highest percentage of true positive results and the lowest percentage of false positive results.

While the present invention is not solely limited to ELISA immunologic techniques, the ELISA technique is particularly preferred since the method allows the artisan to detect concentrations of a particular substance, in this case MMP's, in nanogram per milliliter concentra-tions. Other suitable immunological techniques include radio-immunoassay (RIA), Western dot blot, dip stick, zymography as well as immunologic techniques known to those of ordinary skill in the art. For example, in certain techniques an absolute-type reading, i.e., a color change, indicates the presence of disease. In a preferred embodiment, a quantitative or numeric value indicating the level of enzyme-complex concentration or activity is provided thereby allowing comparison to a reference standard.

In another aspect of the present invention, a kit is provided for detecting the presence of metastatic disease in a biologic sample. The kit provides the inventive diagnostic agent as described herein and means for measuring the total amount of MMP complexed with circulating inhibitors thereof. In particular, the kit includes antibodies, preferably monoclonal or polyclonal antibodies against MMP's and a second type of antibodies, preferably monoclonal or polyclonal antibodies directed against TIMP's. The means for measuring can be any suitable means known to those of ordinary skill in the art such as antibody coated beads or other solid phase immobilized antibodies. Measuring may also be accomplished by Enzyme Linked Immunosorbent Assay (ELISA) radio-immunoassay, zymography and the lik techniques.

Other immunological detection means can be readily adapted for use in connection with the diagnostic agent and method of the present invention. It is intended that all such alternative measuring and diagnostic means be included within the scope of the present invention.

With particular regard to the antibodies included herein, it will be appreciated by those of skill in the art that such antibodies, both monoclonal and polyclonal types are available from commercial sources such as Cell Tech Lmt. of Slough, England, or can be prepared using standard laboratory practices.

EXAMPLES

In the Examples set forth below, various aspects of the present invention are set forth to provide further appreciation of the invention. The Examples, however, are not meant in any way to restrict the effective scope of the invention.

EXAMPLE I

Sandwich-type ELISA Immunoassay For Detection of MMP-2 and MMP-2/TIMP-2 Complexes In Human Plasma

In this Example, a preferred immunoassay is prepared. Initially, 96 well microtiter plates (Dynatech Immulon II, Alexandria, VA) were coated with 100µl of polyclonal rabbit anti-TIMP-2 (NIH, Bethesda, MD) diluted to a concentration of 1:200 in 0.1 M $NaHCO_3$, pH 9.0 for 18 hours at 4°C. The unbound anti-TIMP-2 was removed and 200µl of 1% bovine serum albumin in bicarbonate buffer was added twice for 30 minutes at 27°C to block excess binding sites on the wells. The plate was washed 3 times with a washing buffer containing 50 mM sodium phosphat , pH 7.2, 0.1 M NaCl, 0.05% Tween 20.

Twenty-eight human plasma samples and six 100µl purified MMP-2/TIMP-2 compl x standards were each diluted in an incubation buffer containing 50 mM sodium phosphate, pH 7.2, 0.1M NaCl, 0.02% Tween 20, 0.1% bovine serum albumin, (BSA), and separately added to individual wells for 2 hours at 37°C. The wells were thereafter washed 3 times with washing buffer. Monoclonal murine anti-MMP-2 obtained from the University of Alabama, Birmingham, diluted 1:4000 in incubation buffer, was added in an amount of 100µl per well. The plates were incubated at 37°C for 1 hour.

The plates containing the ELISA sandwich were completed by being washed 3 times and adding 100µl of biotin labeled goat antibodies to mouse IgG, IgA, IgM (H&L chains) in a concentration of 1:1000 before being incubated at 37°C for 1 hour. The plates were again washed 3 times and alkaline-phosphatase conjugated to streptavidin (1:1000 dilution, 100µl) was added to each well and the plates were incubated for 30 min. at 37°C. The biotin and streptavidin reagents are employed to amplify the signal from the detecting antibody reagent, thus permitting the detection of nonogram per ml concentrations of gelatinase-TIMP complexes. Alkaline phosphatase conjugated to streptavidin is the enzyme used to generate color from the p-nitrophenyl phosphate substrate.

The wells were washed 3 times with PBS-Tween phosphate-buffered saline with Tween detergent and 3 times with water. 100µl of substrate p-nitrophenyl phosphate (1mg/ml) in 0.1M glycine, 1mM $MgCl_2$, 1mM $ZnCl_2$, pH 10.4 was added and after 30-90 minutes at room temperature, the plates containing both the human plasma samples and the purified standards were read at A_{405} in a Microplate Autoreader (BioTek EL 309. Winooski, Vt.). Quantification of MMP-2/TIMP-2 complexes was made by extrapolation from a log-log linear regression curve employing varying

conc ntrations of purified MMP-2/TIMP-2 complex as standard. The results are set forth in the Table below and are graphically illustrated in Fig. 1.

TABLE

DIAGNOSIS	STAGE	MMP-2 (ng/ml)	MMP-2/ TIMP-2* (units/ml)	MMP-9 (ng/ml)
		Norm. Conc.= 0-828	Norm. Conc.= 0-694	Norm. Conc.= 0-31
CONTROLS				
A		610	288	0
B		716	458	14
C		677	423	17
D		488	277	18
E		615	271	8
F		823	596	36
G		496	556	1
H		617	188	1
I		1381	604	25
J		633	173	5
K		683	434	2
L		616	446	0
GI CANCER				
A	3	480	1429	63
B	3	774	377	4
C	4	591	657	5
D	4	312	406	22
E	4	664	933	126
F	4	658	596	4
G	4	908	3277	45
H	2	500	779	1
BREAST CANCER				
A	4	622	804	1
B	1	734	509	0
C	3	1145	2428	51
D	3	759	382	4
E	4	786	2429	5
F	4	1226	1278	71
G	4	687	639	41
H	3	734	593	-

Key

*Units/ml based on a reference standard purified from human plasma using gelatin sepharose chromatography followed by gel filtration chromatography.

As shown in the above Table, th normal range was based on th mean ± 2 S.D. f 12 normal plasma samples collected in EDTA anticoagulant. Four out of eight GI cancer patient specimens and four out of eight breast cancer patient specimens (50%) had significantly increased levels of MMP-2/TIMP-2 complexes. These patents tended to have advanced cancer, indicating that this assay would be predictive of patients with metastatic cancer.

The MMP-2/TIMP-2 complex assay provided results that were independent from isolated measurements of either MMP-2 alone or MMP-9 alone. Some patients had elevations of MMP-2/TIMP-2 complexes and normal levels of MMP-2 or MMP-9, indicating that multiple different assays will be supplemental in enhancing the diagnostic utility of these assays. Results of MMP-2 measurements alone have not proven to be useful in the diagnosis of metastatic cancer. Furthermore, it is critical that the blood specimens obtained from patients be anticoagulated such as with EDTA since the levels of MMP's are falsely elevated in serum as a result of release of MMP's especially MMP-9 or gelatinase B from white blood cells during the clotting process.

EXAMPLES II-IX

In these Examples, sandwich ELISA assays were prepared in a manner similar that set forth in Example I, except that the capture and detection antibodies were varied so that different matrix metalloproteinases could be detected. The various combinations of immunoassay detection systems provide the clinician with a battery of diagnostic assays useful in the identification, diagnosis, treatment and determination of prognosis in patients with various types of cancer. The illustrative assays are set forth below.

EXAMPLE	II	III	IV	V	VI	VII	VIII	IX
CAPTURE ANTIBODY TO DETECT TIMP SHOWN	TIMP-1	TIMP-1	TIMP-2	TIMP-1	TIMP-2	TIMP-2	TIMP-2	TIMP-1
DETECTION ANTIBODY TO DETECT MMP SHOWN	MMP-9	MMP-3	MMP-3	MMP-1	MMP-1	MMP-2	MMP-9	MMP-2

The above assays can be combined with other diagnostic methods such as (CEA) carcinoembroyonic antigen measurements useful for colon cancer diagnosis and CA-125 (useful in ovarian cancer diagnosis) as a further or confirmational indicator of tumor growth and/or metastatic disease. It is to be understood that the above listed combinations are illustrative and in no way represent the complete range of possible assays which can be prepared in accordance with the present invention.

EXAMPLE X

In this Example, a sandwich ELISA format immunoassay was prepared in a manner such as that set forth in Example I. The assay in this Example, however, contained mouse monoclonal antibodies to human TIMP-1 MAC-015 antibody from Cell Tech Lmt. to capture TIMP-1/MMP-9 complexes and biotinylated monoclonal mouse antibodies to 92 kDa matrix metalloproteinases prepared acc rding to the meth d set forth

by Bergmann, et al. supra, the disclosure of which is incorporated by reference herein. The assay was used to compare the levels of these complexed antigens present in the plasma of patients with various types of cancer with those of non-cancerous controls. The cancer group was further defined as patients with gastrointestinal cancers (GI) and patients with female genitourinary tract (GU) cancers such as cancers of the ovary, cervix, vagina and uterus. The results are set forth in the Table below.

TABLE

GROUP n=sample size	MMP-9/ TIMP-1 COMPLEX INCREASE ONLY DETECTED	MMP-9 INCREASE ONLY DETECTED	MMP-9/ TIMP-1 COMPLEX AND MMP-9 INCREASE DETECTED	TOTAL NUMBER OF POSITIVES (%)
Controls n=49	1	1	1	3 (6%)
GI cancer n=94	14	14	7	35 (37%)
GU cancer n=23	8	3	4	15 (65%)

Referring now to the Table, it can be clearly seen that there are significant analytical advantages in measuring the levels of MMP complexes in addition to free MMP's to detect or confirm metastatic disease.

The above data dramatically illustrate this point for patients with GU cancer. Consider that by measuring only MMP-9, the clinician was able to confirm the presence of metastatic disease in only 3 of 23 (13%) known metastatic

plasma samples. However, when the clinician also assays complex s of the nzyme and combinati ns of free enzymes and complexes, the summation of all assays provides a much more accurate diagnostic indicator. Moreover, the data obtained from measuring TIMP/MMP complexes is independent from that obtained by measuring MMP-9 assays alone. The present invention, therefore, provides supplemental information regarding metastatic disease. Patients with other types of cancer may have elevations of different types of MMP complexes thus necessitating the performance of a battery of different tests such as that set forth in Examples II-IX to optimize the diagnostic potential of detecting aggressive cancer at an earlier stage.

The above Example provides further evidence of a strong link between the invasive/metastatic process and the increased levels of metalloproteinases, both free and with inhibitor complexes in human plasma. Moreover, there is also a positive correlation between highly metastatic cancer cell lines (phenotypes) and increased secretion of certain enzyme complexes. The practitioner, therefore, can custom tailor the assay, for example, by substituting capture and detection antibodies to measure specific antigens. It is contemplated that assays for specific metastatic diseases such as breast and/or gastrointestinal cancers could be developed based on the results obtained from subjecting a biologic sample to a battery of specific antibodies.

While there have been described what are presently believed to be the preferred embodiments of the invention, those skilled in the art will realize that changes and modifications may be made thereto without the parting from the spirit of the invention, and it is intended to claim all such changes and modifications as fall within the true scope of the invention.

WHAT IS CLAIMED IS:

1. A diagnostic agent for detecting the presence of metastatic activity in a biologic sample, comprising:

a) a first immunologically responsive substance capable of reacting with matrix metalloproteinases; and

b) a second substance capable of reacting with endogenous inhibitors of matrix metalloproteinases.

2. The diagnostic agent of Claim 1, wherein said matrix metalloproteinases are selected from the group consisting of 72 kDa type IV collagenase/gelatinase A (MMP-2), 92 kDa type IV collagenase/gelatinase B (MMP-9), stromelysin (MMP-3), putative metalloproteinase (PUMP) and mixtures thereof.

3. The diagnostic agent of Claim 1, wherein said first responsive substance is capable of reacting with breakdown products of matrix metalloproteinases.

4. The diagnostic agent of Claim 1, wherein said first responsive substance is selected from the group consisting of monoclonal antibodies, polyclonal antibodies and mixtures thereof.

5. The diagnostic agent of Claim 4, wherein said first responsive substance is selected from the group consisting of murine monoclonal anti-human 72 kDa type IV gelatinase antibodies, 92 kDa type IV gelatinase antibodies and anti-human stromelysin and anti-human PUMP and mixtures thereof.

6. The diagnostic agent of Claim 1, wherein said inhibitors of matrix metalloproteinases are selected from the group consisting of tissue inhibitor of metalloproteinase (TIMP-1), tissue inhibitor of metalloproteinase (TIMP-2) alpha-2 macroglobulin and mixtures thereof.

7. Th diagnostic agent of Claim 1, wh rein said second substance is a polyclonal antib dy sel ct from the group consisting of rabbit anti-TIMP-1, rabbit anti-TIMP-2 and anti-alpha-2 macroglobulin.

8. The diagnostic agent of Claim 1, wherein said second substance is a monoclonal antibody to one of TIMP-1, TIMP-2 and mixtures thereof.

9. The diagnostic agent of Claim 5, wherein said monoclonal antibody is selected from the group consisting of antibodies to fragments of gelatinase A or gelatinase B.

10. The diagnostic agent of Claim 1, wherein said first substance is present in an amount of from about 0.01ng/ml to about 2000 ng/ml by weight of said biological sample and said second substance is present in an amount of from about 0.01 ng/ml to about 2000 ng/ml by weight of said biological sample.

11. The diagnostic agent of Claim 10, wherein said first substance is present in an amount of from about 0.01 ng/ml to about 2000 ng/ml by weight of said biological sample and said second substance is present in an amount of from about 0.01 ng/ml to about 2000 ng/ml by weight of said biological sample.

12. A method for detecting the presence of metastatic activity in a biologic sample, comprising:

contacting said biological sample with a diagnostic agent capable of indicating the presence of matrix metalloproteinases (MMP's) and tissue inhibitors of MMPs.

13. The method of Claim 12, wher in said matrix metalloproteinases are selected from the group consisting f 72 kDa type IV collagenase/gelatinase A (MMP-2), 92 kDa type IV collagenase/gelatinase B (MMP-9) and stromelysin (MMP-3) and putative metalloproteinase (PUMP).

14. The method of Claim 12, wherein said first responsive substance is capable of reacting with breakdown products of matrix metalloproteinases.

15. The method of Claim 12, wherein said first responsive substance is selected from the group consisting of monoclonal antibodies, polyclonal antibodies and mixtures thereof.

16. The method of Claim 15, wherein said first responsive substance is selected from the group consisting of murine monoclonal anti-human 72 kDa type IV gelatinase antibodies, 92 kDa type IV gelatinase antibodies and anti-human stromelysin antibodies and anti-human PUMP antibodies.

17. The method of Claim 12, wherein said inhibitors of matrix metalloproteinases are selected from the group consisting of tissue inhibitor of metalloproteinase (TIMP-1), tissue inhibitor of metalloproteinase (TIMP-2) and alpha 2 macroglobulin.

18. The method of Claim 17, wherein said second substance is a polyclonal antibody selected from the group consisting of rabbit anti-TIMP-1, rabbit anti-TIMP-2 and anti-alpha-2 macroglobulin.

19. The method of Claim 12, wherein said second substance is a monoclonal antibody to one of TIMP-1, TIMP-2 and mixtures thereof.

20. The method of Claim 16, wher in said m n clonal antibody is selected from the gr up consisting f antibodies to fragments of gelatinase A or gelatinase B.

21. A kit for detecting the presence of metastatic disease in a biologic sample, comprising:

a) a first means for detecting the presence of matrix metalloproteinases;

b) a second means for detecting the presence of endogenous inhibitors of matrix metalloproteinases thereof; and

c) means for measuring the amount of matrix metalloproteinases detected by said first and said second means.

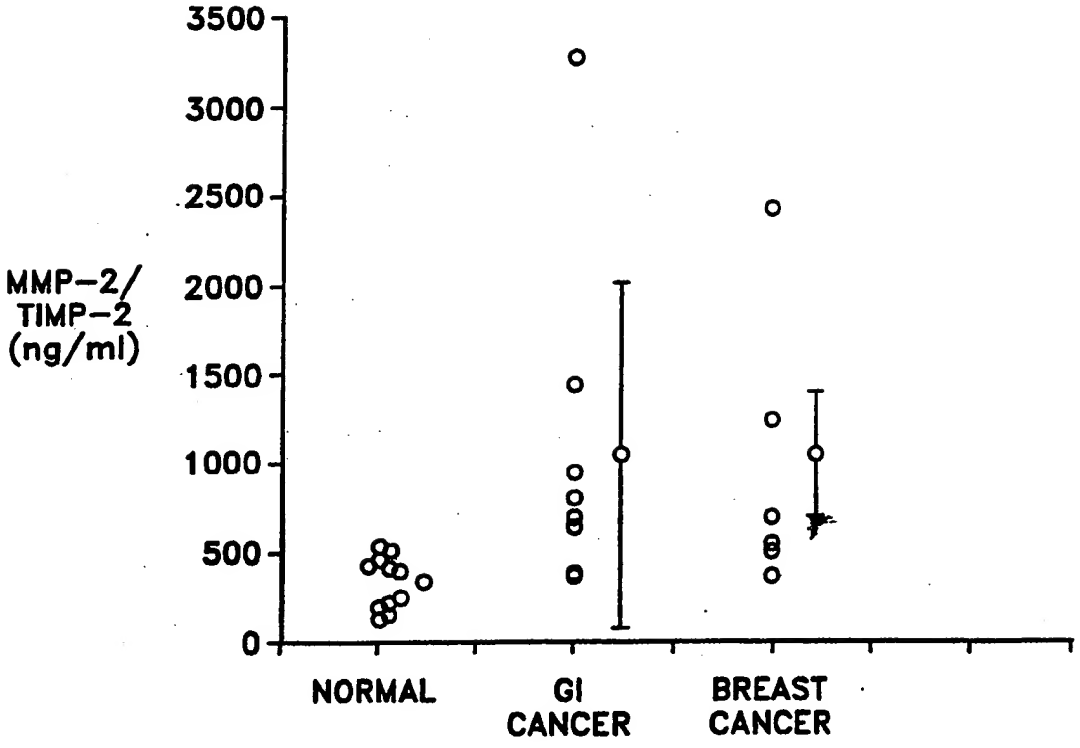
22. The kit of Claim 21, wherein said first means is an antibody against said matrix metalloproteinases and wherein said second means is an antibody against said tissue inhibitors of matrix metalloproteinases.

23. The kit of Claim 21, wherein said means is a monoclonal antibody and said second means is a monoclonal or polyclonal antibody.

24. The kit of Claim 21, wherein said means for measuring is an immunoassay.

25. The kit of Claim 24, wherein said means for measuring is an enzyme linked immunosorbent assay.

FIG—1


3500
3000
2500
2000
1500
1000
500
0
MMP-2/
TIMP-2
(ng/ml)
NORMAL
GI
CANCER
BREAST
CANCER

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02967

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/574; C07K 15/28; C12Q 1/37
US CL :435/7.23, 7.92, 7.94, 23; 530/387.1, 388.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.23, 7.92, 7.94, 23, 810; 436/518; 530/387.1, 388.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	MATRIX, Volume 10, issued 1990, S. Cooksley et al., "Immunoassays for the Detection of Human Collagenase, Stromelysin, Tissue Inhibitor of Metalloproteinases (TIMP) and Enzyme-Inhibitor Complexes," pages 285-291, especially page 285, column 1, first paragraph; page 286, column 1 and column 2, first paragraph.	1, 4, 12, 15 2, 3, 5-11, 13, 14, 16-25

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 JUNE 1993	30 JUN 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer TONI R. SCHEINER
Facsimile N . NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application N .
PCT/US93/02967

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Biol. Chem, Volume 264, number 29, issued 1989, W.G. Stetler-Stevenson et al., "Tissue Inhibitor of Metalloproteinase (TIMP-2)," especially page 17347, column 1, first paragraph and column 2; page 17377, column 2.	2, 3, 5-11, 13, 14, 16-25

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Dialog: Biosis, CAB Abstracts, Medline, Embase, Cancerlit, Derwent
search terms: metasta?, plasma, metalloproteinase, MMP, collagenase, gelatinase, stromelysin, PUMP, macroglobulin, tissue(w)inhibitor, metallophthalein